Grape juice and wine proteins: evolution during fruit ripening. Impact in white wines protein haze and prevention of the risk

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Abstract: Grapevine proteins can play a double role in winemaking. They can have a positive effect in foamability in sparkling wine, but their presence in wine can also be responsible for the wine haze risk (WHR). So, this study is split in two parts as follows: the first part investigated the trend of grape protein content along with the ripening process. Oenological parameters of healthy grapes of cultivars Chardonnay Blanc and Pinot Noir (*Vitis vinifera* L.), vintages 2012, from the Champagne region at different maturity stages, were collected to investigate the relationships between grape berry maturity degree (MD), and other oenological parameters, such as protein content. The results in both varieties showed an increasing trend of total protein contents (analysed by electrophoresis) during the ripening. Strong correlations have been found among grape berry MD and almost all the parameters in both cultivars. The second part of the work, investigated the use of potential alternative to the use of bentonite for colloidal stability. Indeed, clouding and haze formation are serious aesthetic problems especially in white wines which are caused by the presence of flocculated grape proteins. To overcome this problem and stabilize white wines bentonite is widely used, which allows the easy removal of grape proteins. As known bentonite entails many drawbacks, such as loss of wine volume and stripping of aromas. Thus, this study evaluated the use of an alternative treatment to achieve a colloidal stability without the use of bentonite. Aspergillo-pepsins (AGPs) was added in the aim to stabilize a Sauvignon blanc 2017 grape juices from Amboise (Vallée de la Loire - France), with and without heat treatments (75 °C, 1 min) prior to fermentation. Without heating, AGPs showed a low proteolytic activity. When combined with must heating, more than 90% of the total proteins disappeared in both cases (heating carried out before and after enzyme addition) proving in this case that the heating applied alone was sufficient in fact.

Keywords: grape berry maturity; protein; wine; proteases; heat treatment

Resumo: As proteínas da videira podem desempenhar um papel duplo em Enologia. A sua presença pode ter um efeito positivo na formação da espuma em vinhos espumantes, mas pode também estar na origem de turvação indesejada. O presente estudo divide-se em duas partes: na primeira parte é investigada a evolução do teor de proteína ao longo da maturação de uva das variedades Chardonnay Blanc e Pinot Noir (*Vitis vInifera* L.), no decorrer da vindima de 2012. Os resultados obtidos, para ambas as variedades, evidenciaram uma tendência para o aumento do teor de proteína total (analisado por electroforese) durante a maturação. Correlações fortes foram encontrados entre o grau de maturação da uva e quase todos os parâmetros enológicos em ambas as variedades. Na segunda parte deste estudo , foi investigada a utilização de potenciais alternativas ao uso de bentonite para estabilização coloidal. Com efeito, a floculação de proteinas da uva pode resultar na formação de turvação e eventual precipitação no vinho, afectando gravemente o seu aspecto, Actualmente, para ultrapassar este problema e estabilizar os vinhos brancos a bentonite é amplamente utilizada, permitindo a remoção de proteínas de uva. No entanto o tratamento com este auxiliar tecnológico apresenta importantes desvantagens, nomeadamente a perda de volume de vinho e a remoção de compostos do aroma, com perda de intensidade aromática. Assim, foi avaliado um tratamento alternativo para estabilização coloidal sem recurso a bentonite. Concretamente, foi estudada a adição de aspergillo-pepsinas (AGPs, combinada com e sem com e sem tratamento térmico (75 ° C, 1 min), antes da fermentação, em mosto da variedade Sauvignon blanc, com origem em Amboise(Vale do Loire - França), vindima de 2017. Verificou-se que, sem aquecimento as AGP mostraram fraca actividade proteolítica. No entanto, a sua adição conjugada com o tratamento térmico resultou numa diminuição superior a 90% do teor de proteínas totais.

Palavras-chave: maturidade da uva; proteína; vinho; proteases; tratamento térmico

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C	control must
H	Heated must
B10:	must with 10 g/hL bentonite
B20	must with 20 g/hL bentonite
B30	must with 30 g/hL bentonite
B45	must with 45 g/hL bentonite
B60	must with 60 g/hL bentonite
E1	must with 5 mL/hL of Enzyme 1 not heated
E2	must with 5 mL/hL of Enzyme 1 not heated
HE1-5	must with 5 mL/hL of Enzyme 1 added after heating
HE2-5	must with 5 mL/hL of Enzyme 2 added after heating
HE1-2	must with 2 mL/hL of Enzyme 1 added after heating
HE2-2	must with 2 mL/hL of Enzyme 2 added after heating
E1H	must with 5 mL/hL of Enzyme 1 added before heating
E2H	must with 5 mL/hL of Enzyme 2 added before heating
TLPs	taumatine-like proteins
PR	pathogenesis-related proteins
TA	titratable acidity
MD	maturity degree
SDS-PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
MW	molecular weight
OIV	International Organisation of Vine and Wine
AGP	aspergillo-glutamic peptidase
PN	Pinot Noir
CH	Chardonnay Blanc
S1 to S7	maturity stage from S1 to S7
CIEL*a*b*	colour space defined by the International Commission on Illumination (CIE)
$(NH_4)HSO_3$	Ammonium hydrogen sulfite
NaOH	sodium hydroxide
PAC	potential alcohol content
AF	alcoholic fermentation
WHR	wine haze risk
BSA	bovine serum albumin

1. Introduction

1.1 Grape ripening and proteins evolution

Grape berry is a typical true fruit formed by skin, flesh, seeds and a complex vascular system, the growth of this non-climacteric fruit is summarized by the well-known double-sigmoidal curve (**figure 1**) and is divided into an initial and rapid growth, a subsequent lag phase and a second period of growth corresponding to berry ripening (Kanellis and Roubelakis-Angelakis, 1993; Coombe and McCarthy, 2000).

During the first phase, embryo formation takes place in the seeds and the berry enlarges through frequent cell divisions, accompanied by the accumulation of solutes, such as tannins, malic and tartaric acids (Possner and Kliever, 1985; Conde et al., 2007).

The lag phase is characterized by the lack of any changes in berry volume and weight and its end coincides with the onset of ripening. This stage is named veraison and is easily detectable in red cultivars where due to the start of anthocyanins synthesis a change in skin colour takes place. Moreover, at veraison, berries start to soften and this event is mainly linked to significant changes in the cell wall composition (Davies and Robinson, 2000; Nunan et al., 2001).

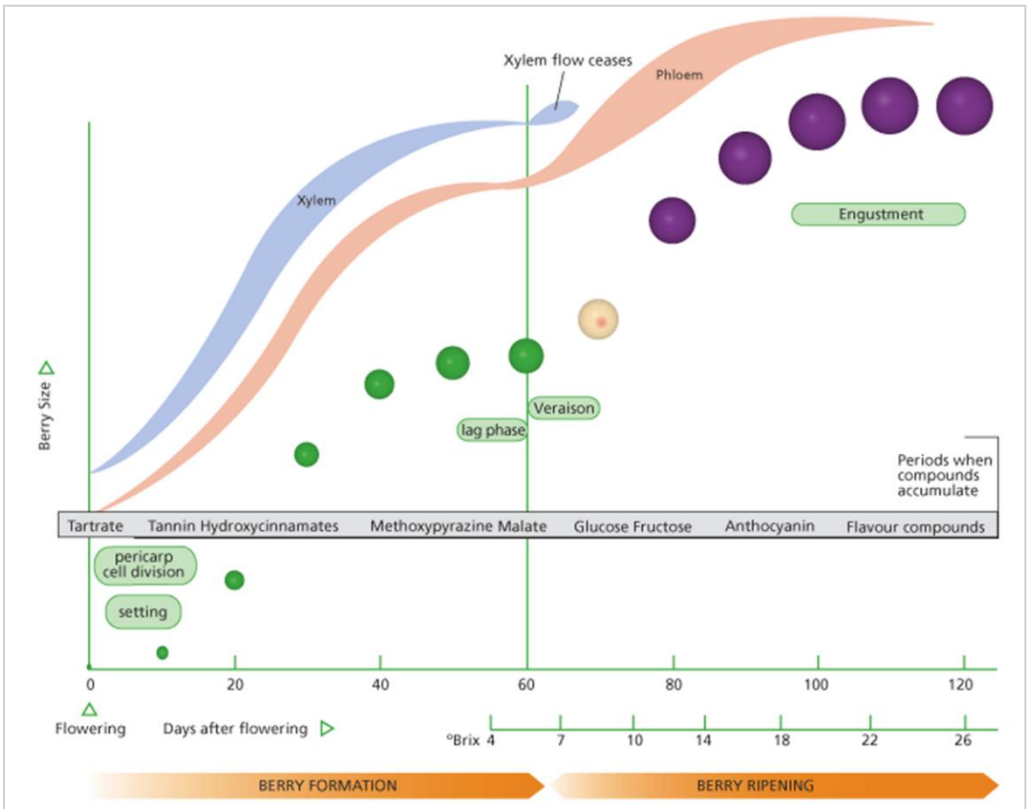

Figure 1. Grape maturation curve (Jordan koutroumanidis, winetitles).

Grape ripening represents the third phase of the double sigmoidal curve of berry development and is characterized by deep changes in berry composition, thus in the organoleptic characteristics. Many of these variations were related to proteins involved in responses to: biotic or abiotic stresser, carbohydrate and amino acid metabolisms.

The major proteins in wine are thaumatin-like proteins (MW around 24 kDa), chitinases (MW around 30 kDa) and invertase (MW around 60 kDa),.

Many of these proteins such as chitinase and thaumatin-like (Vincenzi et al., 2011) but also abscisic stress ripening protein and polyphenol oxidase became the most expressed proteins, furthermore the concentrations of some metabolites, among which malic acid is the most important, decrease while the levels of other molecules, such as glucose, fructose, volatile aroma compounds and anthocyanins in red cultivars greatly increase (Boss and Davies, 2001; Lund and Bohlman, 2006; Conde et al., 2007).

In all growth phases, the very active metabolism of the skin deeply influences the final characteristics of the whole grape berry. The final composition of this tissue depends on both the particular genetic background of the cultivar and the environmental conditions.

This tissue, is formed by a single layer of clear epidermal cells and a few hypodermal layers beneath the epidermis, is in fact the site of the synthesis of anthocyanins and aroma compounds (Boss and Davies, 2001; Adams, 2006; Lund and Bohlman, 2006; Conde et al., 2007) and also represents a fundamental protective barrier against damage by physical injuries and pathogen attacks, even if *Botrytis cinerea* is capable to enter the fruit owing enzymatic activities. (Bargel and Neinhuis, 2005).

1.2 Main oenological parameter evolved over the grape ripening

Among the main parameters which play a central role in establishing the maturity level of the grapes and thus to schedule the harvest and the winemaking process there are:

The sugar content, which is also used to estimate the potential alcohol content (PAC) knowing the rate of the alcoholic fermentation performed by the yeasts which are able to produce 1% v/v of alcohol from 16,83 g/L of sugar (Bindon et al., 2013), even tough, there are many other factors which influence this rate.

Total acidity (TA), according with the International Organisation of Vine and Wine (OIV) includes the complex of fixed acids (tartaric, malic, succinic, lactic, citric) and volatile (which can be removed by boiling, such as acetic acid) present in musts or wines; acidity derived from CO_2 (carbonic acid) should not be included, meanwhile only free forms of SO_2 are considered. More specifically the OIV defines total acidity, as the sum of the titratable acids up to pH 7.0, mean addition of a solution of NaOH (OIV, 2018). The ratio between the sugar and TA is another fundamental parameter which is the grape berry Maturity degree (MD).

The pH is the measurement of the hydrogen ion concentration. This parameter can be measured rather easily. It is an important indicator of the maturity stage of the grapes (James, 1983).

1.3 The importance of grape proteins in grape and in winemaking

In their long association with pathogens, plants evolved an elaborate array of defensive tools. At the same time, those pathogens developed different tools to overcome plant resistance mechanisms as a multimillion year evolutionary ping-pong game (Ownley and Trigiano, 2016). As each defensive innovation was established in the host, new ways to circumvent it evolved in the pathogen generating some of the most complex interactions known to biology (Taylor, 1998). We can consider an interaction between plant and pathogen as an open warfare, whose major weapons are proteins synthesized by both organisms (Ferreira et al., 2001).

Knowledge of the proteins and enzymes present in grapes and their derivate is important to grape juice processors and winemakers essentially because the unstable soluble proteins may precipitate and form hazes and sediments (Hsu and Heatherbell, 1987).

Wine clarity, especially that of white wines, is important to most consumers and is also one of the characteristics that is most easily affected by inappropriate shipping and storage conditions. For this reason, ensure the wine stability prior to bottling is an essential step of the winemaking process and presents a significant challenge for winemakers (Ribéreau-Gayon et al., 2006). Wine protein haze is caused mainly by the aggregation of grape pathogenesis related (PR) proteins, when a wine is heated for a period of time, either during transport or under regular storage conditions over long periods of time. Proteins in the wine slowly unfold and aggregate, causing a fluffy precipitate, making the wine look cloudy with a milky aspect (**Figure 2**) and unappealing to consumers (Waters et al., 2005). Protein haze can also be mistaken for microbial spoilage or tartrate instability (Ferreira et al., 2001). A stable white wine is one that is clear and free from precipitates at the time of bottling, through transport and storage, to the time of consumption (Steven et al., 2015). These proteolytic resistant proteins were identified as (PR) proteins, namely chitinases and thaumatin-like proteins (TLPs) (Waters et al., 1996). Further studies examined the behaviour of these proteins during wine-haze formation and identified chitinase as the major cause of protein haze (Falconer et al., 2010).

Figure 2. Clear white wine and turbid wine caused by protein aggregation.

However, the same proteins involved in protein haze in wine are also involved in the foaming proprieties of the sparkling wines as shown in many studies indicating a positive correlation between protein content and foamability in grape juices (López-Barajas et al., 1997) and wines (Brissonnet and Maujean, 1993; Martínez-Lapuente et al., 2015).

Since the ability of sparkling wines to form a stable collar is considered by consumers to be a criterion of quality there is a great interest in understanding the factors affecting the foamability of wine (Brissonnet and Maujean, 1993).

It seems that foam stability is significantly favored by the presence of surface active agents which can stabilize foam by settling at the bubble's surface, their hydrophobic head turned towards the gas, and their hydrophilic tail turned towards the aqueous phase (Bamforth, 1985). And among the various foam active substances, proteins and some polysaccharides seem to play a major role because of their surface properties. (Brissonnet and Maujean, 1991,1993; Abdallah et al., 2010).

More in detail, it has been reported that thaumatin-like proteins and invertase are involved in promoting positive sparkling wine foam properties whereas chitinases do not seem to have any effect. But these proteins as previously said such as thaumatin-like proteins, β-(1,3)-glucanases and chitinases, are the main involved in protein haze formation. (Waters et al., 2005; Esteruelas, 2009).

Although other components in wine can also have an effect on foam characteristics. the literature on the subject is usually inconclusive and sometimes contradictory. (Brissonet and Maujean, 1991; Dussaud et al., 1994; Pueyo et al., 1995; López-Barajas et al., 1997). To eliminate the protein haze risk, often there is the necessity to treat white wines with bentonite (Pocock and Waters, 2006). Obviously, by removing some of the foam active proteins,

bentonite can negatively affect the foaming properties of sparkling wines (Martínez-Rodríguez and Polo, 2003; Dambrouck et al., 2005; Vanrell et al., 2007).

Furthermore, due to its capacity to secrete proteases *Botrytis cinerea* has the capacity to lower the wine foamability by degrading the proteins involved in this phenomenon, which can completely disappear in the infected one. (Marchal et al., 1998,2001; Cilindre et al., 2007; Cilindre et al., 2008). The juice of grapes infected with *Botrytis cinerea* was found to have significantly lower concentrations of PR proteins than juice from healthy grapes (Marchal et al., 1998; Girbau et al., 2004). One particular protease from *B. cinerea*, BcAP8, has proven to be effective against grape chitinases during juice fermentation without the need for heating.

When BcAP8 was added to juice prior to fermentation, the resulting wines produced significantly less heat-induced protein haze than wines made without BcAP8 (Van Sluyter et al., 2013). Since plants continuously evolve ways to inhibit pathogen growth, and vice versa, pathogens continuously evolve ways to counteract the inhibitory effects of PR proteins (Bishop et al., 2000), recent investigations have focused on grape pathogens for specificity against PR grape proteins in order to use these proteases as benefit for winemakers worldwide (Steven et al., 2015).

1.4 The mechanisms of haze formation

The proteins consist of nitrogen bodies that can be separated by electrophoresis (Koch and Sajak, 1959).

The most abundant classes of haze-forming proteins that occur in grape (*Vitis vinifera*) juice and white wines are chitinases and thaumatin-like proteins (**Figure 3**) (Waters et al., 1996; Pocock et al., 2000; Vincenzi et al., 2011).

These proteins are small (<35 kDa) and compact, have globular structures, are positively charged at wine pH, and are tolerant of low pH in juice and wine (Ferreira et al., 2001; Waters et al., 2005). Moreover, chitinases and TLPs have a high number of disulphide bonds that contribute to the highly stable globular structures of these proteins (Hamel et al., 1997; Marangon et al., 2014). The mechanisms associated with haze formation in wines are not well understood and yet commonly cited as a two-stage process. Shortly, in the first stage, wine proteins unfold in response to stimuli such as elevated storage temperatures and higher temperatures leading to more rapid protein unfolding (Dufrechou et al., 2010; Falconer et al., 2010; Marangon et al., 2011; Sauvage et al., 2011; Gazzola et al., 2012; Dufrechou et al., 2013).

Once unfolded, the proteins aggregate and flocculate to form a visible haze (Dufrechou et al., 2013) without SO_2 proteins refold and do not produce haze. When proteins unfold, they expose

hydrophobic binding sites that are generally buried in the core of the proteins, and more hydrophobic proteins tend to cause hazes more easily, this suggests that the aggregation stage of haze formation is likely to be driven by hydrophobic interactions (Marangon et al., 2010). In addition to differing aggregation behaviours of different wine proteins, other components of wine can also contribute to haze formation. These components include polyphenols, sulfate, (Pocock et al., 2007) and polysaccharides in particular, as well as characteristics of the wine matrix such as wine pH and organic acids which have been attributed a positive effect in wine protein stability (Batista et al., 2009,2010; Dufrechou et al., 2013).

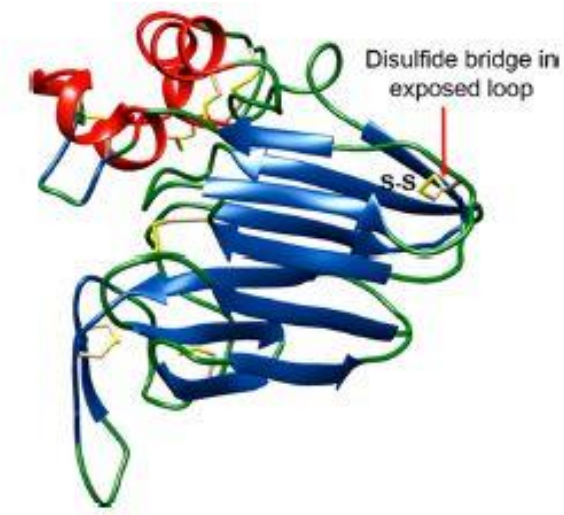

Figure 3. Representation of a heat-unstable thaumatin-like protein. In yellow is possible to see the disulphide bonds. The arrow indicates an exposed disulphide bond that could be susceptible to reduction by heat and sulphites (semanticscholar.org).

1.5 Protein stability

Among the different phenomena of instability that can occur in a wine, the turbidity due to protein haze represents one of the most important and considered issue to deal with, especially in white wines. However, also in red wines, especially those young ones because of the tannic concentration, it can be a critical parameter to analyse in order to prevent unwanted protein precipitations after bottling. As written in the definition of the OIV (OIV, 2018) turbidity is the reduction of the transparency of a liquid due to the presence of undissolved substances. Its unit of measurement is the NTU Nephelometric Turbidity Unit, which is the value corresponding

to the measurement of the light diffused by a standard suspension at a 90° angle to the direction of the incident beam.

To check the degree of stability of a wine it is necessary to apply a reliable method that is well adapted to the substrate which is the heat test (20 mL of clear wine is heated up and maintained in a water bath at temperature of 80 °C for 30 min). the turbidity of the wine is measured by a nephelometer prior and after the heat treatment. Once the sample has been cooled down, its turbidity is checked again and compared with the initial value. If the difference between the turbidity of the initial sample and after the heat passage is more than 2, it means that the wine is unstable and a treatment must be considered; if the value is less than 2 no treatment will be required (International Organization of Vine and Wine - O.I.V. Paris, France, 2009). There are many other heat tests based on a different rate temperature-time which not always give the same results (Pocock et al. 1973; Waters et al. 1992).

It is also important to say that the heat test, may overestimate the risk that a particular wine will have by denaturing both haze-forming and non-haze forming proteins. This can over predict the amount of bentonite needed to stabilize the wine, leading to less cost-effective winemaking practices. Thus, in order to develop new strategies for haze prevention in white wines it is necessary to understand the mechanisms of haze formation and the structures of the proteins involved in this phenomenon (Pocock and Waters, 2006).

1.6 Strategies for wine haze prevention

Since haze-forming proteins become cations at wine pH, while at the low pH bentonite, a montmorillonite clay is negatively charged, it is widely used as treatment to remove the positive charged proteins.

Unfortunately, this procedure lowers the wine quality because of the stripping of aromatic compounds (Miller et al., 1985; Puigdeu et al., 1996). In addition, a significant loss in wine volume occurs as a result of the bentonite lees formation (Voilley et al., 1990).

Once understood the mechanisms of protein haze formation in wine, there are several possible strategies for preventing wine haze that would eliminate or at least reduce the need for bentonite, such as the following (Steven et al., 2015).

1. decreasing the ionic strength of the wine;
2. decreasing the polyphenol concentrations in wine;
3. stabilizing wine proteins against thermal unfolding;
4. disrupting hydrophobic protein−protein interactions;
5. degrading wine proteins enzymatically;
6. using alternative adsorbents or ultrafiltration to remove proteins.

Strategies 1 and 2, using industrial-scale electro dialysis, ion exchange, and fining technologies, are impractical because they would dramatically change wine sensory attributes. Strategies 3 and 4 are potentially related in practice, as the promotion or addition of specific glycoproteins/polysaccharides, including specific yeast mannoproteins, could both stabilize wine proteins and interfere with hydrophobic protein−protein interactions (Dufrechou et al., 2012).

The ability of yeast mannoproteins to stabilize wine proteins was attributed specifically to the glycan portion of mannoprotein (Schmidt et al. 2009). However, the main problem of these practices is that it is unclear what level of protection against haze formation is possible achieve through the use of polysaccharides (Butzke et al., 2012).

The most promising alternative strategies to bentonite are the strategy 5 scilicet to degrade wine proteins with enzymes and strategy 6 which is the development of novel fining agents. The use of enzymes in order to degrade haze forming proteins in wine is a particularly appealing alternative to bentonite because it allows to minimize the losses of wine volume and the aroma stripping which is unavoidable using bentonite. Ideally, effective enzymes would be added to grape juice or ferment without the need for later removal, such as already happens with pectinases (Moreno-Arribas et al., 2005).

Moreover, the yeasts may utilize the degradation products of grape proteins as nitrogen sources and this obviously would potentially reduce the need for nitrogen additions (Guitart et al., 1999; Pretorius, 2000).

The two types of enzymatic activities relevant to wine protein degradation are: 1) the reduction of disulphide bonds by protein disulphide reductases and 2) the hydrolysis of peptide bonds by proteases. Protein disulphide reductases could, theoretically, precipitate haze-forming proteins during winemaking throughout the reduction of disulphide bonds, because the chemical reduction of disulphide bonds has been shown to facilitate the unfolding of these proteins. (Marangon et al., 2010).

However, there is scarcity of publications regarding the use of protein disulphide reductases active under winemaking conditions. For this reason, the search for wine-relevant enzymes to degrade haze-forming proteins has focused on proteases (Pavlenko et al., 1969).

The difficulty in using proteases for degrading haze forming proteins is the stability of the proteins in wine-like conditions. Especially chitinases and TLPs are highly resistant to proteases in their native state due to their structure (Waters et al., 1992, 1995, Tattersall et al., 2001) and so they are able to tolerate the endogenous proteases that degrade many grape proteins already during the first phases of the grape processing (Waters et al., 1996).

It has been developed a new method that involves heating grape juice in the presence of a heat-tolerant proteases prior to fermentation to produce wine that is free from haze-forming

proteins (Marangon et al., 2012). In brief, when juice is heated the proteins unfold and become susceptible to enzymatic activity.

The obvious possible drawbacks of this method is the exposition of grape juice or wine to high temperature which could have negative sensory implications and the requirements of high energy inputs (Urlaub, 1986; Lloyd et al., 2005). Even though it has been shown that negative sensory changes can be contained, as well as the energy requirements by optimizing the combination temperature-treatment time (Marangon et al., 2012).

Indeed, some research has focused on the ideal temperature and time of heating required to unfold haze forming proteins containing the damages toward aromatic compounds (Pocock et al., 2003).

However, this method (Marangon et al., 2012) is based on a flash pasteurization, that means a rapid heating of grape juice to 75 °C for 1 min. The use of this technique also requires the addition of a protease that is active at the low values of pH which are the one of grape juice and white wines (pH 2.9−3.5) and at 75 °C. Among the different proteases, the Aspergillo-glutamic peptidase (AGP), has been found to be active at these condition, and adding AGP to clarified grape juice prior to flash pasteurization and fermentation resulted in wines that were heat stable and almost completely free from haze-forming proteins. Furthermore, chemical and sensory analysis indicated that there were no significant changes to the main parameters or wine sensory profile on the wines treated (Marangon et al., 2012). This treatment has been shown to be effective at industrial scale, (Robinson et al., 2012) inasmuch as the cost of this treatment compared with bentonite treatment, making it a potentially cost-effective and commercially viable bentonite alternative (Marangon et al., 2012).

2 Aim of the work

The present thesis work is split in two main branches with grape proteins as a common topic. The aim of the first part of this work was the evaluation of the maturity on the grape oenological parameters, focused mainly on the protein composition and concentration of must obtained from Pinot Noir and Chardonnay Blanc grape varieties, two widely spread grape varieties in Champagne region (Experiment 1 - **Chardonnay Blanc and Pinot Noir**).

The aim of second part of my work was the evaluation of the effectiveness of different treatment applied on Sauvignon blanc must in order to remove or at least reduce the use of bentonite to prevent the protein haze risk (Experiment 2 - **Sauvignon blanc**).

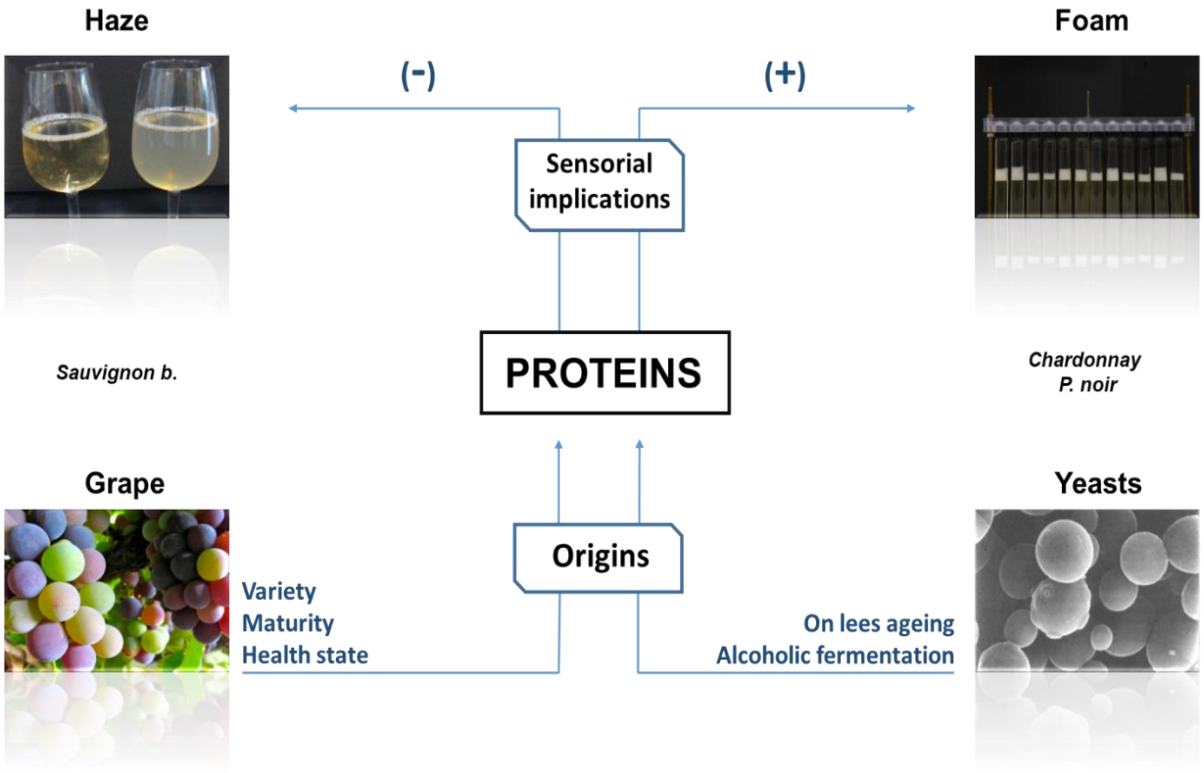

Figure 4. Origins and sensorial implications of proteins in wine.

3. Materials and methods

3.1 Experiment 1 - Evolution of protein composition and concentration during maturation of Pinot Noir and Chardonnay Blanc grapes

3.1.1 Grapes and juice production

In 2012 Pinot Noir (PN) and Chardonnay Blanc (CH) healthy grape clusters (*Vitis vinifera* L.) from the Champagne region were hand-harvested at different maturity levels. Seven stages of maturity for Chardonnay Blanc (indicated as S1 to S7) and 5 for Pinot Noir (S1 to S5).
The dates of the harvest are given in **Tables 1 and 2**. For example, S1 (22/08) means that the sample 1 was harvested the 22nd of August. After the harvest, the grape clusters were stored at -80 °C. One night before the beginning of the pressing they were defrost at 4 °C and then, just before the pressing the samples were brought all at same temperature of 18 °C.
The bunches were pressed without destemming with a mini vertical basket-press whose capacity is 250 g (University of Reims Champagne-Ardenne, Reims, France).
For each stage of maturity, the juice corresponding to the different squeezes of a pressing cycle were collected and blended.
The yield of the first squeeze was 52% of the weight, while 10% for the second, so in total the yield was 62%, which represent the yield observed at industrial scale. Between the two pressing cycles, grape clusters were decompressed without trituration.
Liquid sulfur dioxide was immediately added to all the grape juices at the concentration of 80 mg/L.

3.1.2 Physical-chemical analyses

The analytical methods recommended by the Compendium of International Methods of Wine and Must Analysis (OIV, 2018) were used to determine the pH, titratable acidity (g/L H2(SO4)), sugars (g/L), PAC (v/v) of grape juices. The grape berry maturity degree (MD) was calculated as the ratio of sugars to titratable acidity.
A CRISON® pH-meter (University of Reims Champagne-Ardenne, Reims, France) was used to determine the pH. The TA was determined by NaOH titration, using bromothymol blue as an indicator. An Anton Paar DMA 35 Density Meter was used to analyze the density and then it has been converted in sugar, according to the mass per volume unit. The PAC is derived from sugar content, and the result is based on the assumption of the following performance of yeast: 16.83 g/L of sugar produces 1% v/v of alcohol.
After one night of static settling at 18 °C, 100 mL of each grape juice sample was centrifuged (15 min at 4500 g, 18 °C). The supernatant was separated, then directly analyzed for

oenological parameters (basic physical-chemical parameters) which are reported **in table 1** and **2**.

After filtration through a 0.45 *µ*m membrane filter, the Pinot Noir samples, according to the method OIV (OIV, 2018) have been analyzed for the absorbance at 420, 520 and 620 nm, (SHIMADZU® UV-1800 spectrophotometer) and for determination of chromatic characteristics CIEL*a*b* using A Lovibond® PFX190 Tindometer Series II (**Table 3**).

3.1.3 Wine protein analysis

SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis), was used to determine the protein compositions and quantifications of the must in this study, according to the method of Laemmli (Laemmli, 1970). The 8.3 cm × 7.3 cm dimension and 1.0 mm thick slab gel was composed of 4% w/v polyacrylamide (Bio-Rad, USA) stacking gel and 13% polyacrylamide separating gel. Electrophoretic analysis has the ability to determine protein concentration and assess protein fractions by their molecular weights.

A vertical Mini-PROTEAN®III electrophoresis apparatus (Bio-Rad, USA) was used to run the gel at a constant voltage of 150 V until the bromophenol blue tracker dye reached the gel bottom. The samples were mixed with the 4X Laemmli buffer (v/v = 3:1) and 18 *µ*L of the mixture was loaded in each well. Five-fold diluted standard proteins from 10 to 250 kDa (Precision Plus Protein TM Unstained Standards, Bio-Rad, USA) were used as MW markers and 1 *µ*L was loaded. The MWs of wine proteins were calculated from the linear regression equation of log MW versus mobility. After migration, gels were silver-stained according to the protocol described by Rabilloud (Rabilloud et al., 1994).

For each sample, the gels have been carried out four times.

After silver nitrate coloration, the SDS-PAGE gels were scanned with a Bio-Rad Doc XR+ scanner and analyzed using the Image Lab software.

3.2 Experiment 2 - Evaluation of protein composition and concentration of Sauvignon Blanc wine treated by heating, bentonite at different concentration and enzymes.

3.2.1 Production of juice

In 2017 Sauvignon Blanc variety vines (*Vitis vinifera* L.) from Amboise (Vallé de la Loire, France) were harvested mechanically. Then the grape berries were pressed with a pneumatic press. Suddenly after the pressing, SO_2 at the concentration of 4 g/hL and pectolytic enzymes

have been added in the must. After 16 h at 16 °C in order to allow the settling, the must was racked. Then 15 different treatments were applied on the must of Sauvignon blanc in triplicate in 375 mL glass bottles. In order to better understand the winemaking protocol, all of the different modalities of treatments are reported in detail in the **Figure 5**.

The heat treatment has been performed by immersion of the bottles in a 95 °C water able to rapidly (10 min) bring the grape juice until a temperature of 72 °C. The must was kept at 72 °C for 1 minute and then quickly (5 min) cooled at the temperature of 50°C using tap water at 12 °C. The two different enzymes employed were both Aspergillo-pepsin and were indicated as E1 and E2. These enzymes were added in the must before heating at the concentration of 50 μL/L, meanwhile in the must after heat treatment/cooling at a dose of 20 and 50 μL/L (enzyme concentration given by the manufacturer unknown).

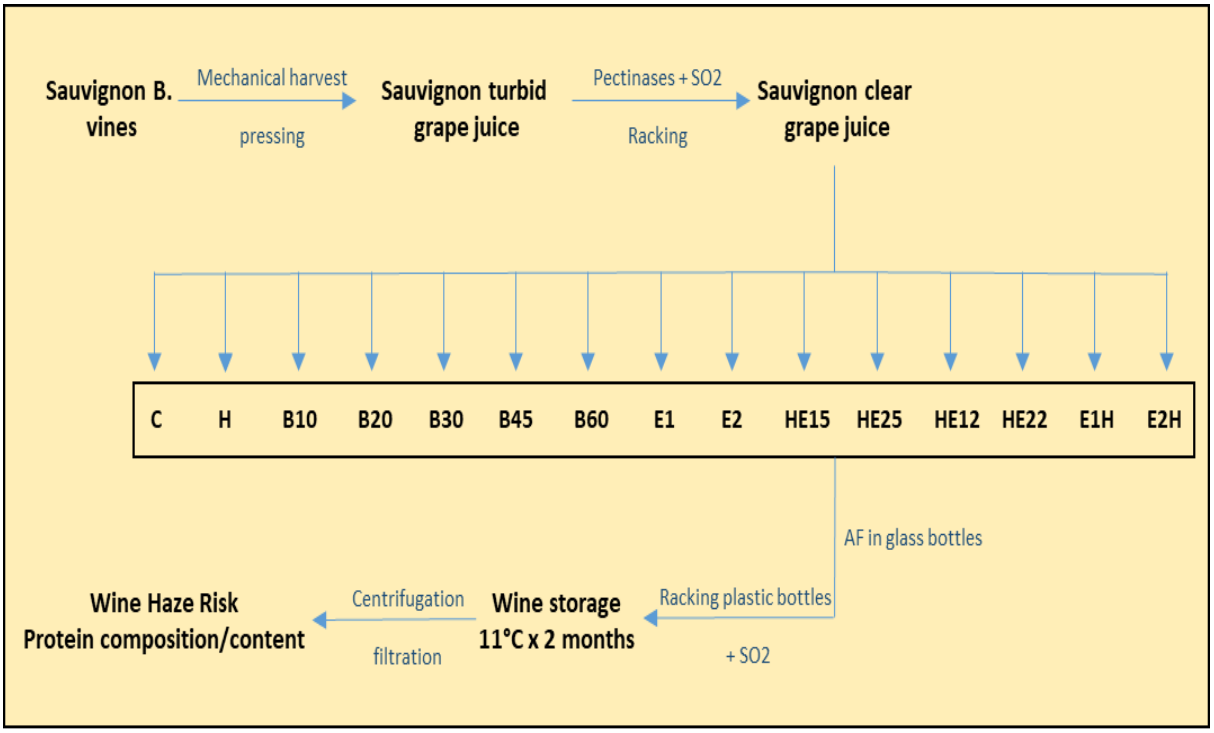

Figure 5. Detailed winemaking protocol of Sauvignon Blanc: **C**: Control must, **H**: Heated must, **B10**: must with 10 g/hL bentonite, **B20**: must with 20 g/hL bentonite, **B30**: must with 30 g/hL bentonite, **B45**: must with 45 g/hL bentonite, **B 60**: must with 60 g/hL bentonite, **E1**: must with 5 mL/hL of Enzyme 1 not heated, **E2**: must with 5 mL/hL of Enzyme 1 not heated, **HE1-5**: must with 5 mL/hL of Enzyme 1 added after heating, **HE2-5**: must with 5 mL/hL of Enzyme 2 added after heating, **HE1-2**: must with 2 mL/hL of Enzyme 1 added after heating, **HE2-2**: must with 2 mL/hL of Enzyme 2 added after heating, **E1H**: must with 5 mL/hL of Enzyme 1 added before heating, **E2H**: must with 5 mL/hL of Enzyme 2 added before heating.

3.2.2 Alcoholic fermentation

The alcoholic fermentation (AF) of the 15 modalities treated musts in triplicate has taken place directly in the bottle. The beginning of the AF was triggered by inoculation with the yeasts 200 mg / L of LSA (Levulia®GC, Oenolia). The AF was performed in air conditioned room at 18 °C and it lasted 12 days.
Once the AF was completed sulphur dioxide was added at the concentration of 80 mg/L, the wine was racked in plastic bottles and stored in anoxic conditions for 2 months at 11 °C.

3.2.3 Wine haze risk assessment

The wine samples were filtered with a 45 µm membrane. 20 mL of each sample was put in a glass tube and then the turbidity was measured using a turbidimeter before and after the temperature was raised up at 80 °C for 30 min using a Julabo® TWB bath in order to check the haze risck.

3.2.4 Wine protein analysis

In this experiment (Sauvignon blanc), two different techniques were used to determine the total protein concentration of base wines. The SDS-PAGE (with the same conditions used for CH and PN) and a modified Bradford method (Marchal et al., 1997).
The modified Bradford method was used in order to avoid the interferences due to ethanol and phenolic compounds. In brief, the wine protein reactivity with the Coomassie Blue Brilliant is equal to the difference between wine and ultra-filtrate reactivities with the dye reagent respectively. Wines were ultra-filtrated with Amicon® Ultra-4 (3 kDa MWCO, Merck Millipore, Ireland) and the ultra-filtrate was recovered. The assay was carried out as follows: 200 µL of Bradford dye reagent (Bio-Rad, USA) was added to 400 µL of sample (wine or ultrafiltrate) plus 400 µL of ultrapure water. Absorbance of the mixture was determined at 595 nm after 30 min of reaction. Results were expressed in mg/L equivalent to bovine serum albumin (BSA) which was used as a standard. Each value was the average of three independent measures.

3.3 Statistical analysis

The correlation test was performed in both the experiments using Microsoft Office Excel 2016 software. This test allowed to obtain the correlation coefficients in order to reveal the relationships among oenological parameters (pH, TA, sugar content, PAC, grape berry MD) and total protein content (Bradford and SDS-PAGE quantifications).

4. Results and discussion

The main results of the analysis performed are reported below separately for each experiment: Chardonnay Blanc and Pinot Noir grape juices at different grape berry maturity levels (First section) and Sauvignon Blanc (second section).

4.1 Experiment 1 – Evolution of protein composition and concentration during maturation of Pinot Noir and Chardonnay Blanc grapes

4.1.1 Basic physical-chemical characteristics of grape juices

The oenological parameters determined for Chardonnay Blanc and Pinot Noir grape juices at different maturity levels are shown in **Tables 1**, **2** and **3** respectively.

Sugar content is an important indicator of grape berry maturity stage. Inasmuch as it allows to estimates PAC it is the main parameter used to establish the harvest date in winemaking industry. For both CH and PN grapes an efficient sugar accumulation among the maturity levels was observed, which is in accord with the typical grape berry sugar evolution previously reported (Bindon et al., 2013; Lasanta et al., 2014).

An increased trend was found in the pH of grape juices along with the maturity level. Meanwhile TA level decreased with grape ripening mostly due to the catabolism of the malic acid (Lasanta et al., 2014). The grape berry MD has been defined as the ratio sugar/TA.

The highest values were observed for the last sampling in both cultivars.

Table 1 also indicated Chardonnay Blanc MD varying from 2.9 in S1 to 29.9 in S7, but the gain in terms of MD between the stage S6 and S7 is only 0.1 so it remained almost unchanged. The explanation of this stability is likely due to the high heterogeneity of ripening among the bunches and at the selection of healthy bunches that were at the sampling moment the less ripe of the field. The same situation can be observed for Pinot Noir between the S3 and S4 (**Table 2** and **3**) and not only for MD but even for other parameters such as pH, absorbance and the CIEL*a*b* coordinates.

Table 1. Oenological parameters analyzed in 2012 Chardonnay Blanc grape juices at different stage of maturity.

Chardonnay Blanc grape juice 2012

Parameters	**St 1 (30/08)**	**St 2 (07/09)**	**St 3 (14/09)**	**St 4 (21/09)**	**St 5 (28/09)**	**St 6 (05/10)**	**St 7 (12/10)**
sugar g/L	55.3	104.0	133.0	167.9	168.9	193.0	187.6
TA (H_2SO_4) g/L	19.3	14.2	9.8	7.2	7.2	6.5	6.3
pH	2.6	2.8	2.9	2.1	3.0	3.1	3.0
MD	2.9	7.4	13.6	23.4	23.5	29.8	29.9
PAC % vol	3.3	6.3	8.01	10.1	10.2	11.6	11.3

TA - titratable acidity; MD - maturity degree; PAC - potential alcohol content.

Table 2. Oenological parameters analyzed in 2012 Pinot Noir grape juices at different stage of maturity.

Pinot Noir grape juice 2012

Parameters	**St 1 (22/08)**	**St 2 (04/09)**	**St 3 (10/09)**	**St 4 (17/09)**	**St 5 (17/09)**
sugar g/L	54.8	119.4	157.3	150.4	181.2
TA (H_2SO_4) g/L	19.6	11.1	9.1	8.6	6.6
pH	2.69	3.0	3.1	3.1	3.3
MD	2.8	10.8	17.3	17.5	27.5
PAC % vol	3.3	7.2	9.5	9.5	10.9

Abbreviations used: TA, titratable acidity; MD, maturity degree; PAC, potential alcohol content.

Table 3. Color parameters analyzed in 2012 Pinot Noir grape juices at different stage of maturity.

Pinot Noir grape juice 2012

Parameters	**St 1**	**St 2**	**St 3**	**St 4**	**St 5**
A_{420} *nm*	0.167	0.195	0.273	0.273	0.359
A_{520} *nm*	0.053	0.179	0.279	0.298	0.405
A_{620} *nm*	0.004	0.012	0.023	0.023	0.036
*L**	89.258	78.403	70.638	70.099	63.394
*a**	2.9	24.9	34.8	36.8	44.2
*b**	28.3	24.0	28.8	29.2	35.1

4.1.2 Wine proteins

Wine protein concentration and composition was assessed by SDS-PAGE (Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis).

From the first to the last maturity level the general trend was an increase of the total protein content in the must, whatever the cultivar. According to a previous study (Dambrouck et al., 2005) most wine proteins come from grape berries, and some are released by yeast during alcoholic fermentation. In this study, inasmuch as was analysed only the grape juice the increment in protein content is probably due to the ripening process, the variety and environmental and health conditions.

This result was then confirmed by the observed significant correlation coefficients between grape MD and the total protein content, which in both the cultivars Chardonnay Blanc and Pinot Noir has shown an R^2=0.96 (**Tables 4** and **5**).

Moreover, total protein content also showed high correlation to other grape maturity parameters, such as pH and sugar content.

As shown in **Figure 7** and **Figure 8**, the protein bands of CH and PN grape juice were distributed in a wide range of MWs varying from 250 to 10 kDa.

What is also important to highlight is that in both cases in Chardonnay Blanc and Pinot Noir gels (**Figures 7** and **8**) the S1 has shown a very dark lane from the top to the bottom. This line

is probably due to the polyphenols and thus it is considered as interference which could lead toward wrong values and results.

F**igures 9** and **10,** report the results of total protein content assessed by SDS-PAGE in Chardonnay Blanc and Pinot Noir varieties.

The S1 higher value is likely due to the interference by the phenols, for this reason the correspondent columns have been evidenced in green.

For this reason, the S1 values were not used for the further statistical analysis.

Even though, as said above the general trend of both cultivars was an increase of the total protein content, the **Figures 9** and **10** show how the behaviour in terms of increasing in protein content is substantially different between the two cultivars.

Probably seen the higher intensity of the bands in PN even the total protein content is higher, but to confirm that further studies are required, at least for 3 vintages.

Nevertheless, as shown in **Figure 6**, there are not differences in terms of protein composition between Chardonnay Blanc and Pinot Noir samples.

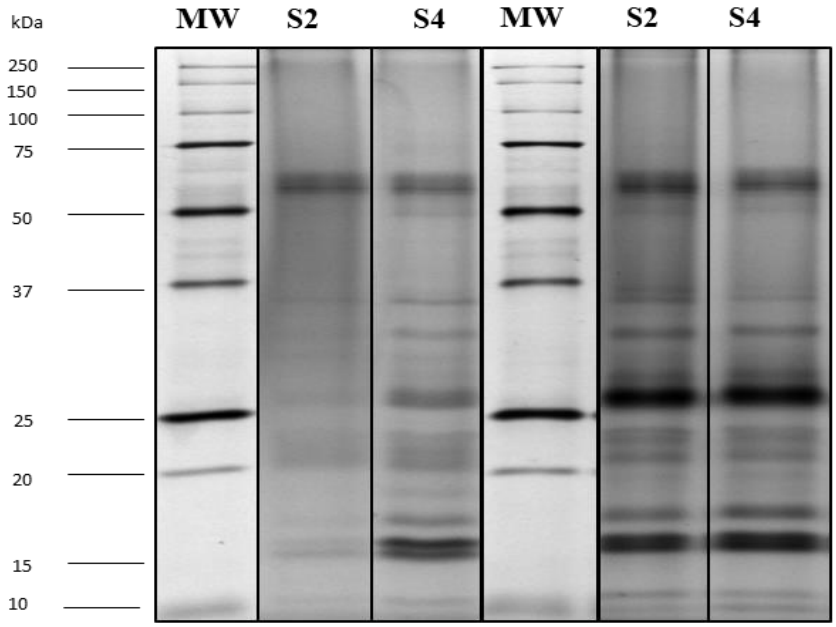

Figure 6. Comparison of protein composition between Chardonnay Blanc (on the left) and Pinot Noir (on the right) 2012. MW, molecular weight; S2, maturity stage 2; S4, maturity stage 4.

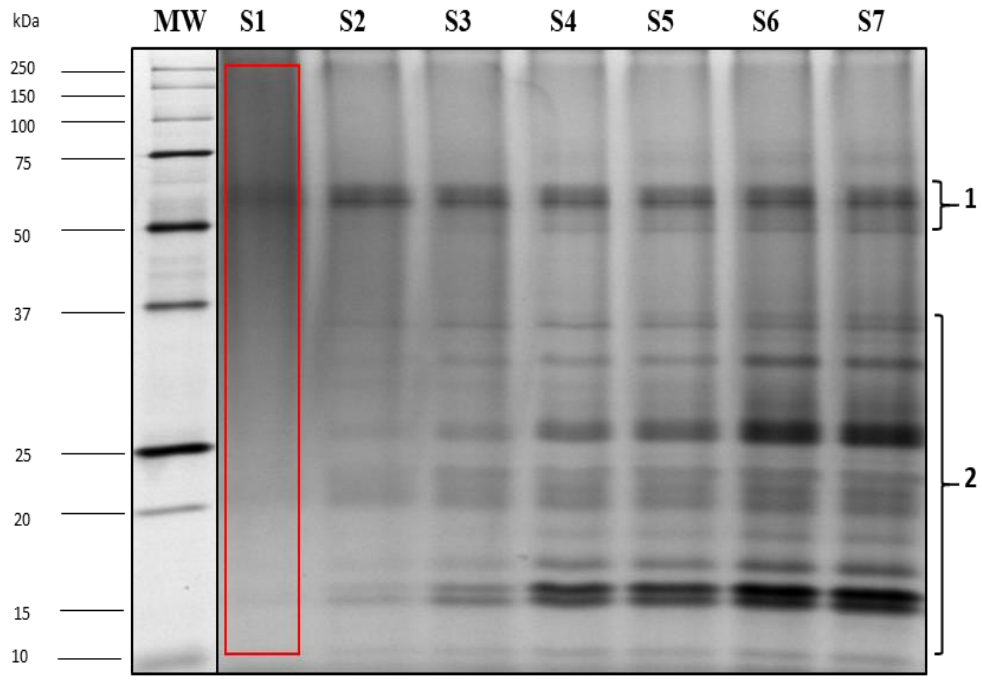

Figure 7. Protein composition (SDS-PAGE + $AgNO_3$) of Chardonnay Blanc 2012 (MW: molecular weight marker), the red rectangle indicates the S1 sample which shows the interference by polyphenols.
MW, molecular weight; S1 to S7, maturity stages.

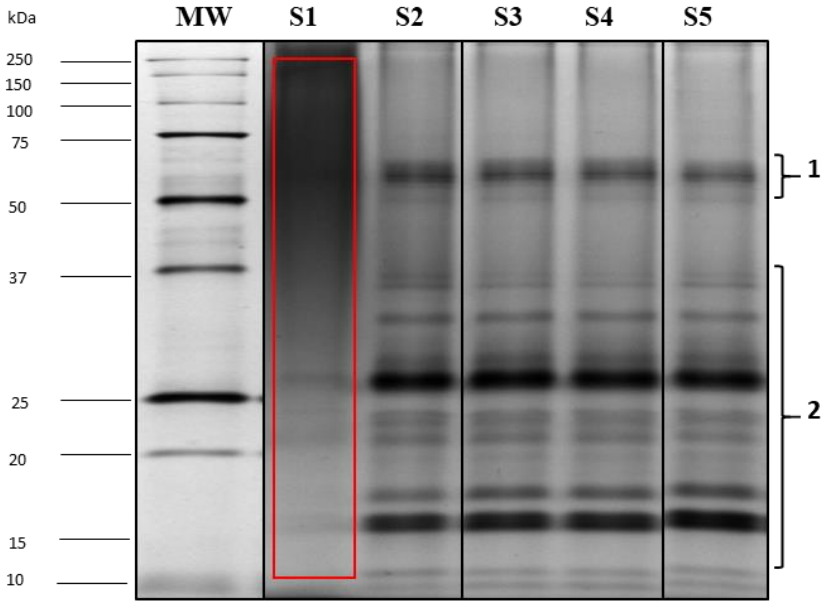

Figure 8. Protein composition (SDS-PAGE + $AgNO_3$) of Pinot Noir 2012, (MW: molecular weight marker), The red rectangle indicates the S1 sample which shows the interference by polyphenols.
MW - molecular weight; S1 to S5 - maturity stages.

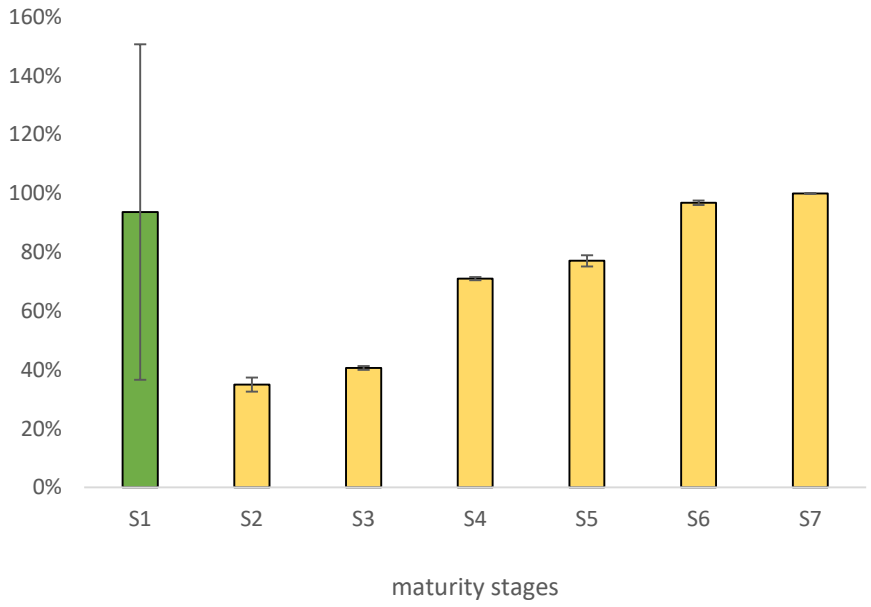

Figure 9. Total protein content of Chardonnay Blanc grape must obtained by SDS-PAGE from S1 to S7. The error bars represent the standard deviation.

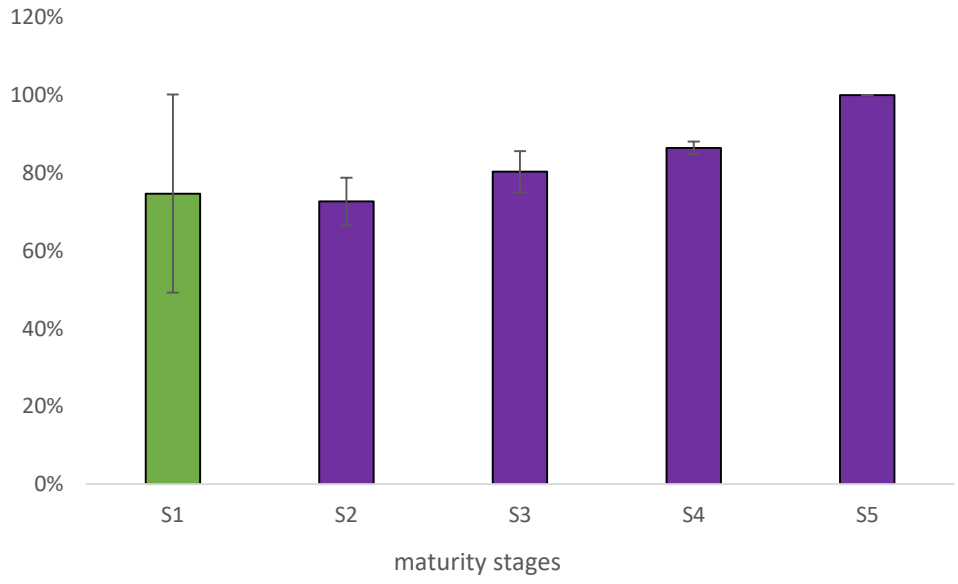

Figure 10. total protein content of Pinot Noir grape must obtained by SDS-PAGE from S1 to S5. The error bar represent the standard deviation.

4.1.3 Correlation analysis

Correlations among all the data were calculated to find out if parameters were statistically correlated (**Tables 4** and **5**). These correlations can be classified as follows: s*ignificant correlation* with $R^2 > 0.95$, and *high correlation* with $0.95 < R^2 < 0.8$. In both cases the gels were separated in two blocks (**block 1** and **2**) in order to analyse separately the behaviour of the two main groups of protein during the ripening (**Figures 7** and **8**).

For the Chardonnay Blanc were found 4 indicated significant correlations and 16 high correlations. Meanwhile for Pinot Noir were found 44 significant correlations and 33 highly correlated parameters.

Table 4. Correlation coefficients R^2 between all the parameters of 2012 Chardonnay Blanc grape juices analyzed. The units of measure used are: sugar content g/L, titratable acidity (TA) g(H_2SO_4)/L; total prot. indicates the total protein content expressed in percentage, meanwhile block 1 and 2 represent the protein content of block 2 and block 3 respectively; Maturity degree (MD) has been obtained by the ratio sugar content/titratable acidity.

Parameters	sugar cont.	TA	pH	MD	block 1	block 2	Tot prot.
sugar cont.	1				*$R^2 > 0,95$ significative correlation*		
TA	0.93	1			*$0,95 < R^2 < 0,8$ high correlation*		
pH	0.987	0.914	1				
MD	0.994	0.905	0.966	1			
block 1	0.923	0.863	0.885	0.932	1		
block 2	0.915	0.863	0.872	0.932	0.888	1	
Tot Prot.	0.937	0.7768	0.898	0.964	0.911	0.911	1

Table 5. Correlation coefficients R^2 between all the parameters of 2012 Pinot Noir grape juices analyzed. The units of measure used are: sugar content g/L, titratable acidity (TA) g(H2SO4)/L; Total prot. Indicates the total protein content expressed in percentage, meanwhile block 1 and 2 represent the protein content of block 1 and block 2 respectively; Maturity degree (MD) has been obtained by the ratio sugar content/titratable acidity. 420 nm, 520 nm, 620 nm represent the values of absorbance; L* a* b* are the coordinate which define a color space.

Parameters	sugar cont.	TA	pH	MD	block 1	block 2	tot prot.	420 nm	520 nm	620 nm	L*	a*	b*
sugar cont.	1									$R^2 > 0,95$ significative correlation			
TA	0.945	1								$0,95 < R^2 < 0,8$ high correlation			
pH	0.969	0.921	1										
MD	0.935	0.969	0.968	1									
block 1	0.893	0.855	0.976	0.948	1								
block 2	0.786	0.926	0.827	0.939	0.822	1							
tot prot.	0.846	0.966	0.860	0.958	0.831	0.991	1						
420 nm	0.974	0.987	0.972	0.988	0.921	0.901	0.94	1					
520 nm	0.952	0.999	0.937	0.98	0.878	0.928	0.966	0.993	1				
620 nm	0.968	0.984	0.974	0.993	0.93	0.91	0.945	0.999	0.992	1			
L*	0.977	0.992	0.943	0.962	0.867	0.877	0.928	0.992	0.993	0.987	1		
a*	0.955	0.991	0.901	0.936	0.811	0.872	0.929	0.973	0.986	0.966	0.994	1	
b*	0.956	0.990	0.96	0.993	0.916	0.929	0.961	0.997	0.996	0.998	0.986	0.97	1

4.2 Experiment 2 - Evaluation of protein composition and concentration of Sauvignon Blanc wine treated by heating, bentonite at different concentration and enzymes.

4.2.1 Heat test results

A heat test (30 min at 80 °C) was applied on the grape juice after centrifugation and filtration. After 2 h at room temperature, the turbidity was higher than 80 NTU. So, we have estimated that this grape juice was a nice candidate for the experiment.

The grape juice heat treatment at 72 °C induced a decrease of the wine haze risk (WHR) by 96% of the sample H that became stable showing 1 NTU. This WHR was pretty equal to the bentonite treated samples between 30 and 60 mg/L which have shown a turbidity level of 0.5 NTU (**Figure 11**).

The dose of bentonite considered as sufficient to obtain a stable wine was 30 g/hL.

If proteases were used without heating (E1, E2), the haze test at 80 °C gave values higher than the control wine because the enzymes flocculated during the haze test, thus increasing the haziness. All of the other treatments with addition of both the enzymes gave a WHR lower than 1,4 NTU. The heat treatment applied to unfold grape berry proteins was in this case sufficient to eliminate the WHR. This result was unexpected when considering the grape juice

haze risk. Nevertheless, all of the wines heated were stable, with or without enzymes. So the study loses all interest.

Further experiments will study the possible relationships between grape juice composition/haze risk and the wine composition/haze risk. Then, it will be possible to better know if a grape juice heat treatment or a heat treatment plus enzymes is necessary to obtain a stable wine from the colloidal point of view.

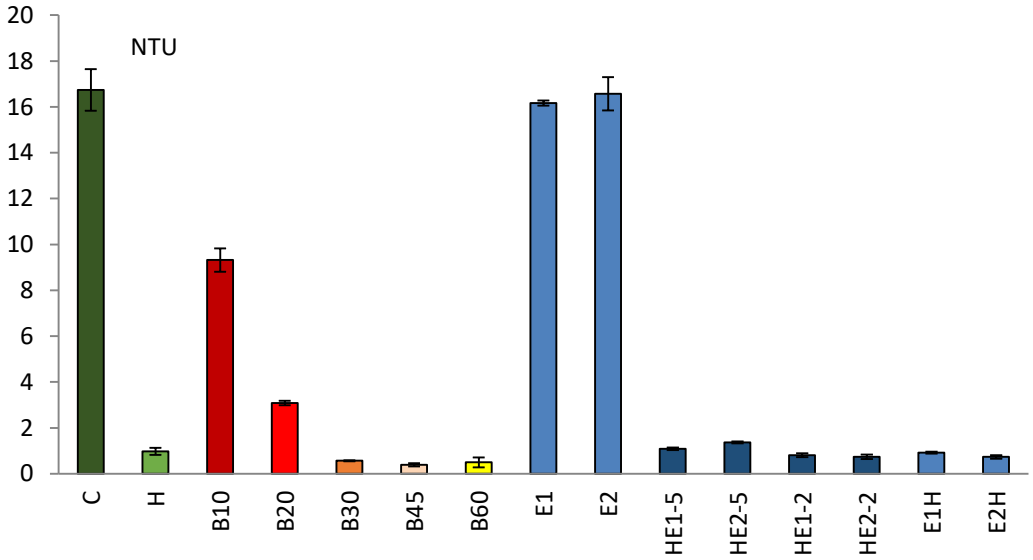

Figure 11. Wine Haze Risk on Sauvignon Blanc estimated by heat test of 30 min at 80 °C.
C: Control must, **H**: Heated must, **B10**: must with 10 g/hL bentonite, **B20**: must with 20 g/hL bentonite, **B30**: must with 30 g/hL bentonite, **B45**: must with 45 g/hL bentonite, **B 60**: must with 60 g/hL bentonite, **E1**: must with 5 mL/hL of Enzyme 1 not heated, **E2**: must with 5 mL/hL of Enzyme 1 not heated, **HE1-5**: must with 5 mL/hL of Enzyme 1 added after heating, **HE2-5**: must with 5 mL/hL of Enzyme 2 added after heating, **HE1-2**: must with 2 mL/hL of Enzyme 1 added after heating, **HE2-2**: must with 2 mL/hL of Enzyme 2 added after heating, **E1H**: must with 5 mL/hL of Enzyme 1 added before heating, **E2H**: must with 5 mL/hL of Enzyme 2 added before heating.

4.2.2 Protein content of single blocks

The gel obtained by SDS-PAGE technique allows to better explain the heat test results.

As shown in the **Figure 12**, the gel has been virtually separated in four blocks from 1 to 4 depending by the MW.

For each block it has been performed a densitometric integration which has given the relative protein content of each block in order to better understand the behavior of each group of protein when undergone to different treatments.

The scanned electrophoresis gel confirmed that the lowest protein content was noted for H and B30 and all of the samples for which proteases were added before and/or after heating and refreshing at 50 °C.

Moreover, this image allows to confirm that, when proteases have been used without heating (E1), the haze test gave values equal than the control wine because the enzymes flocculated during the haze test and forms the new bands in E1 and HE1-5. Even though a slight reduction in intensity was observed in E1 in block 3 and 4. This means a probable effect of the enzyme even at fermentation temperature.

The **Figures 13**, **14**, **15** and **16** show the protein content of each corresponding blocks expressed as a percentage. The error bar represents the standard deviation associated to the mean value of 3 replicates.

The **Figure 13** contains information regarding the proteins with a MW between 55 and 70 kDa. The intense stained band visible in this block is probably due to the invertase which has a MW of 62-64 kDa. The invertase level remains almost unvaried in all of the modalities due to its high heat stability (Kotoyoshi and Koki, 1990).

The only treatment where it is possible to see a lower intensity of the whole band is for the bentonite treated samples B10, B20 and B30. In these cases, the bentonite allows its removing (instead of its disruption), this is also confirmed by the lower values of proteins found in the block 1 (**Figure 13**).

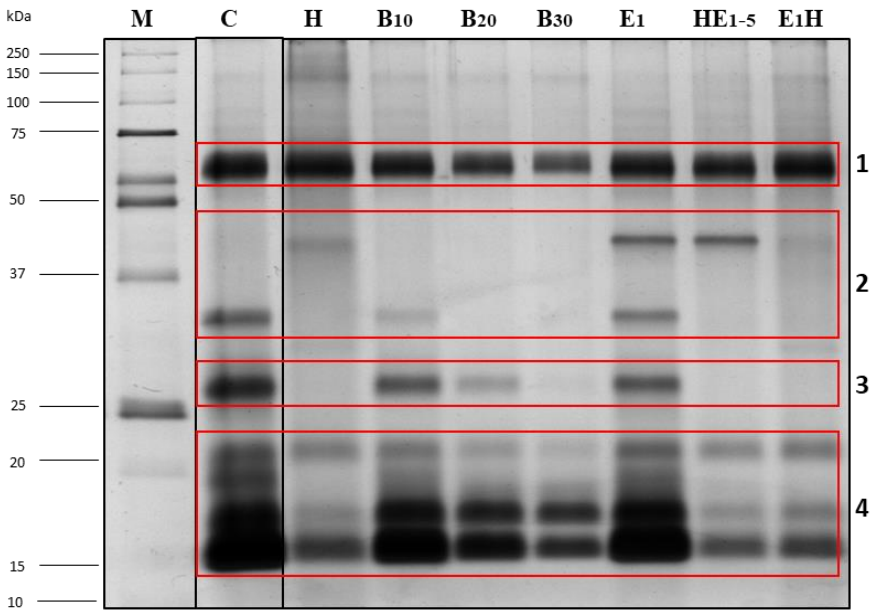

Figure 12. Protein composition (SDS-PAGE + $AgNO_3$) of Sauvignon Blanc 2017.
M: marker, **C:** Control must, **H**: Heated must, **B10**: must with 10 g/hL bentonite, **B20**: must with 20 g/hL bentonite, **B30**: must with 30 g/hL bentonite, **E1**: must with 5 mL/hL of Enzyme 1 not heated, **HE1-5**: must with 5 mL/hL of Enzyme 1 added after heating, **E1H**: must with 5 mL/hL of Enzyme 1 added before heating.

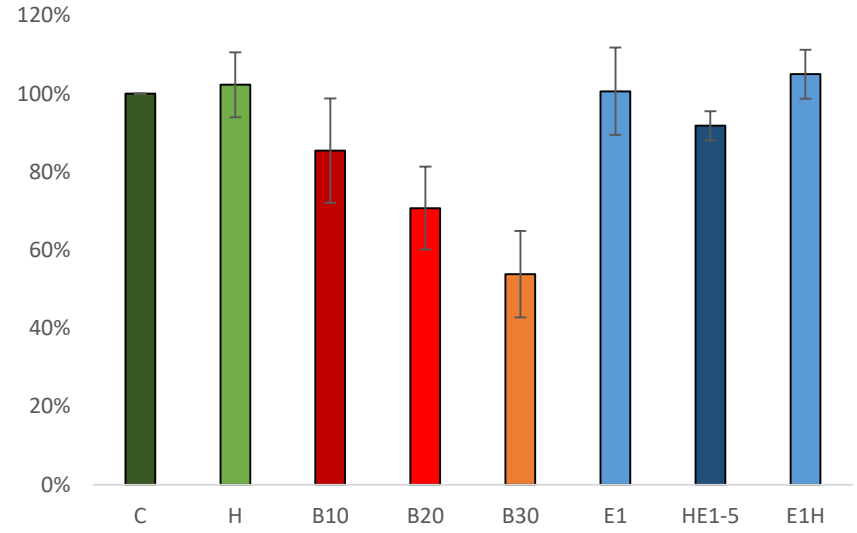

Figure 13. Protein content Block 1 Sauvignon Blanc obtained by SDS-PAGE + $AgNO_3$.
C: Control must, **H**: Heated must, **B10**: must with 10 g/hL bentonite, **B20**: must with 20 g/hL bentonite, **B30**: must with 30 g/hL bentonite, **E1**: must with 5 mL/hL of Enzyme 1 not heated, **HE1-5**: must with 5 mL/hL of Enzyme 1 added after heating, **E1H**: must with 5 mL/hL of Enzyme 1 added before heating.

In the block 2 (**Figure 14**) the protein content in the sample B10, B20 and B30 is lower than the control wine. The E1 and slightly also HE1-5 and E1H have given a higher protein level compared with the control and bentonite treated, likely due to the addition of the enzyme which increase the protein content. This hypothesis is in accord with the band present only in E1 and HE1-5 and E1H (**Figure 12**), due probably, properly to the presence of the enzymes.

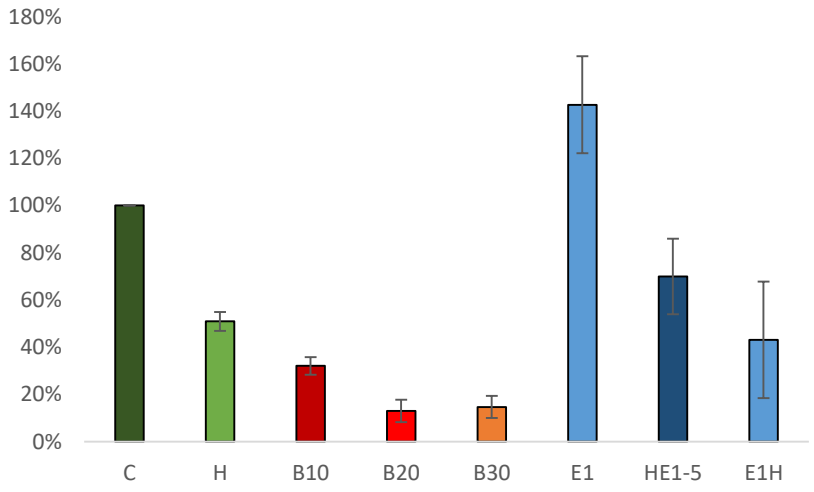

Figure 14. Protein content Block 2 Sauvignon Blanc obtained by SDS-PAGE + $AgNO_3$.
C: Control must, **H**: Heated must, **B10**: must with 10 g/hL bentonite, **B20**: must with 20 g/hL bentonite, **B30**: must with 30 g/hL bentonite, **E1**: must with 5 mL/hL of Enzyme 1 not heated, **HE1-5**: must with 5 mL/hL of Enzyme 1 added after heating, **E1H**: must with 5 mL/hL of Enzyme 1 added before heating.

The block 3 (**Figure 15**) shows high differences in terms of protein content.

A decrease between 94 and 97% have been found in H, B30, HE1-5 and E1H compared with the control wine. This block ranges from 25 until around 30 kDa which is the range in which belong the chitinases, which together with thaumatin-like proteins (TLPs) are the two predominant PR protein families present in finished wine (Waters et al., 1996,1998; Pocock et al., 2000) and the mainly responsible for haze formation in white wines (Waters et al., 1996; Ferreira et al., 2001).

So probably these heat unstable proteins, were flocculated by the heat treatment, thus explaining the lower values in H, HE1-5 and E1H, either they are removed increasingly by bentonite as shown from B10 to B30.

Meanwhile, in E1 we have a higher value probably because the enzyme was not able to efficiently attack the highly stable structures of these proteins (Hamel et al., 1997; Marangon et al., 2014) when juice is not heated. Without denaturation, the proteins do not unfold and they are not susceptible to enzymatic activity (Marangon et al., 2012).

Even though a slight reduction of protein content was observed comparing E1 and C, this supports the hypothesis made that probably there is a little effect of the enzyme even at fermentation temperature.

Exactly the same reasoning can be made for the block 4 (**Figure 15**) which includes proteins with a MW lower than 25 kDa, typical of the main PR protein causing haze in wines (Waters et al., 1996; Ferreira et al., 2001).

Even in this case indeed, E1-5 has sown a value lower than the control wine.

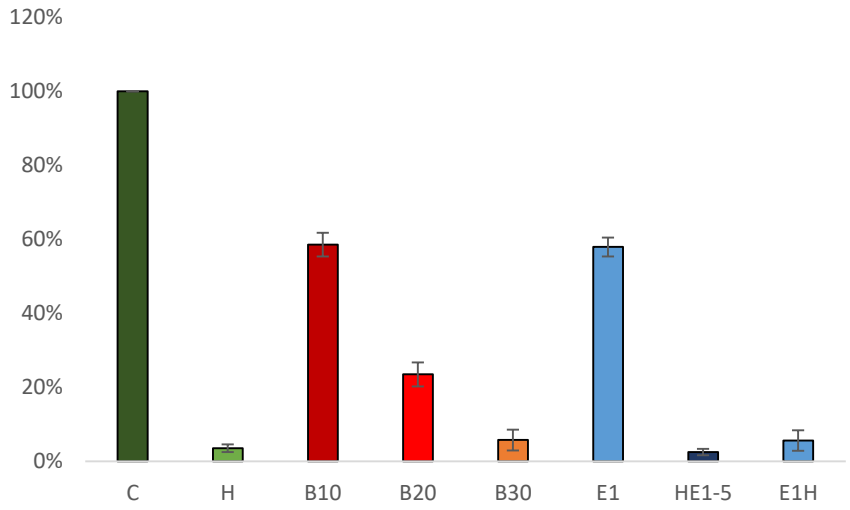

Figure 15. Protein content Block 3 Sauvignon Blanc obtained by SDS-PAGE + $AgNO_3$.
C: Control must, **H**: Heated must, **B10**: must with 10 g/hL bentonite, **B20**: must with 20 g/hL bentonite, **B30**: must with 30 g/hL bentonite, **E1**: must with 5 mL/hL of Enzyme 1 not heated, **HE1-5**: must with 5 mL/hL of Enzyme 1 added after heating, **E1H**: must with 5 mL/hL of Enzyme 1 added before heating.

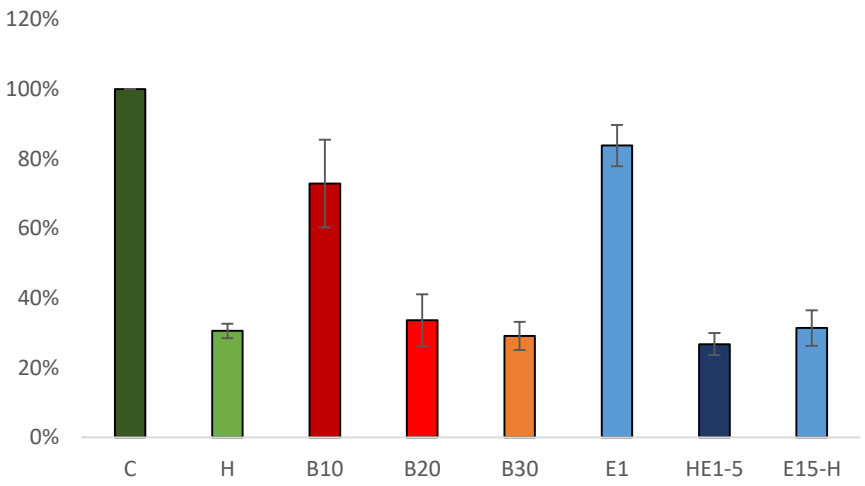

Figure 16. Protein content Block 4 Sauvignon Blanc obtained by SDS-PAGE + $AgNO_3$.
C: Control must, **H**: Heated must, **B10**: must with 10 g/hL bentonite, **B20**: must with 20 g/hL bentonite, **B30**: must with 30 g/hL bentonite, **E1**: must with 5 mL/hL of Enzyme 1 not heated, **HE1-5**: must with 5 mL/hL of Enzyme 1 added after heating, **E1H**: must with 5 mL/hL of Enzyme 1 added before heating.

4.2.3 Total protein content

In this study, the total protein content of base wines was 1) determined by a silver-stained SDS-PAGE which is more sensitive and allows the study of wine proteins without any pre-treatment, and 2) analysed by a modified Bradford method.

By the modified Bradford method, polyaminoacids with MWs more than 3 kDa were all measured and regarded as total protein content (Sedmak and Grossberg, 1977).

Both methods have their own characteristics and advantages.

The Bradford method is faster and low cost, while SDS-PAGE could give more information concerning the protein composition.

Between the two protein quantification methods, significant correlations were shown (R^2>0.96). It indicates that these two quantifications could support each other.

Both **Figures 17 and 18** show how with heat treatment with and without enzyme addition it is possible to achieve quantity of total protein similar than B30, which resulted to be the most effective treatment to lower the protein content.

Furthermore, in both cases the E1 showed a protein content higher or equal to the control which is in accord with both results of the heat test and the band detected in the block 1 of the gel due to de presence of the enzymes.

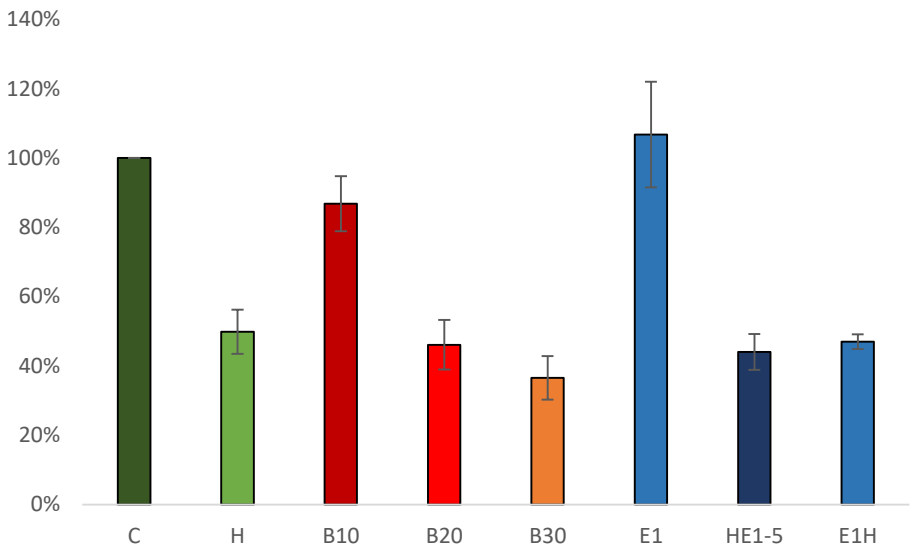

Figure 17. Total protein content Sauvignon Blanc obtained by SDS-PAGE + $AgNO_3$.
C: Control must, **H**: Heated must, **B10**: must with 10 g/hL bentonite, **B20**: must with 20 g/hL bentonite, **B30**: must with 30 g/hL bentonite, **E1**: must with 5 mL/hL of Enzyme 1 not heated, **HE1-5**: must with 5 mL/hL of Enzyme 1 added after heating, **E1H**: must with 5 mL/hL of Enzyme 1 added before heating.

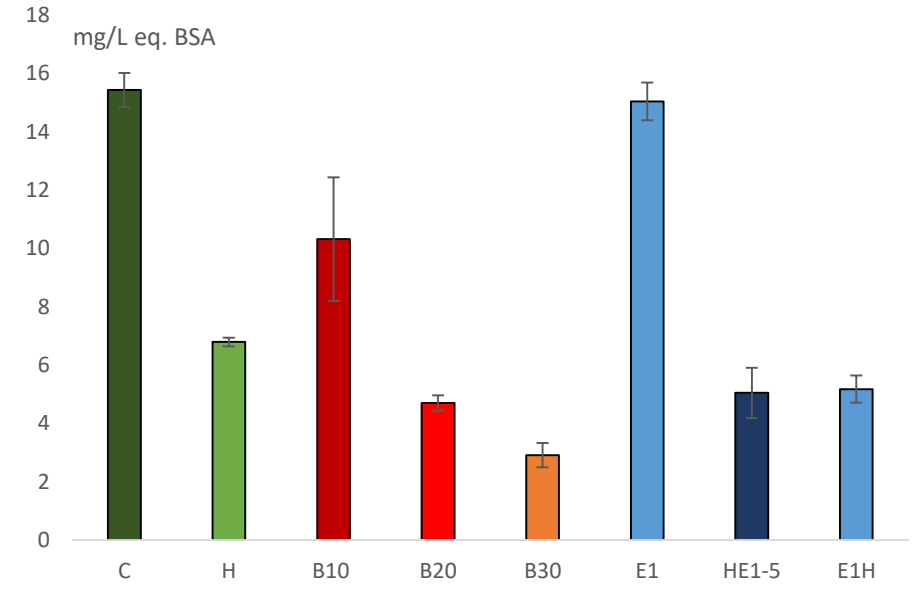

Figure 18. Protein concentration Sauvignon Blanc obtained by modified Bradford method.
C: Control must, **H**: Heated must, **B10**: must with 10 g/hL bentonite, **B20**: must with 20 g/hL bentonite, **B30**: must with 30 g/hL bentonite, **E1**: must with 5 mL/hL of Enzyme 1 not heated, **HE1-5**: must with 5 mL/hL of Enzyme 1 added after heating, **E1H**: must with 5 mL/hL of Enzyme 1 added before heating.

Table 6. Correlation coefficients R^2 of 2017 Sauvignon Blanc wine among the: Wine Haze Risk, whole protein content estimated by Bradford method, total protein content estimated by SDS-PAGE + $AgNO_3$ staining (indicated in the table as total intensity), the protein content of each block indicated as block 1,2,3 and 4. Block 23 is the protein content of block 2 + block 3; Block 34 is the protein content of block 3 + block 4; block 234 is the protein content of block 2 + block 3 + block 4.

	bradford	heat test	Int total	block 1	block 2	block 3	block 4	block 2 3	block 34	block 234
bradford	**1**							*R^2>0.95 significative correlation*		
heat test	0.9451	**1**						*95<R^2<80 high correlation*		
Int total	0.9635	0.9535	**1**							
block 1	0.2817	0.1207	0.1978	**1**						
block 2	0.6608	0.5665	0.5664	0.4053	**1**					
block 3	0.8134	0.8915	0.8188	0.0635	0.2947	**1**				
block 4	0.86	0.9133	0.9584	0.0769	0.4364	0.7842	**1**			
block 2 3	0.9567	0.9719	0.9157	0.187	0.6182	0.8944	0.8225	**1**		
block 34	0.8852	0.9459	0.9649	0.0777	0.4186	0.8658	0.9884	0.8738	**1**	
block 234	0.9214	0.9656	0.9835	0.1077	0.5016	0.8481	0.9852	0.9052	0.9931	**1**

5. Conclusion and further studies

Grape berry maturity and the grape sanitary status influence many oenological parameters of grape juice such as sugar content, pH, total acidity, grape berry MD and particularly protein content and composition. Not surprisingly, these parameters are closely related to grape ripening state.

Inasmuch as the composition of the grape juice is a fundamental requirement able to affect the final wine quality, a great attention should be payed to the maturity stage especially in cool region for the production of sparkling wines (such as champagne) due the capacity of proteins to affect the foamability of base wine as demonstrated by many studies (for Review, Kemp et al., 2018).

In this experiment the general trend was the increase of protein content along to the ripening, thus under the premise of guaranteed grape health, delaying harvest date is an oenological decision that could improve base wine foamability. But it is also true that this increasing trend in protein content was mainly due to the increment of protein fractions with MWs approximate between 31.9 and 17.7 kDa which includes the proteins mainly responsible for the protein haze in wine (Waters, et al., 1996; Ferreira, et al., 2001).

On the other hand, indeed there is a big problem concerning proteins, which is the negative influence of the proteins in still wine or even more in hotter region producing sparkling wines such as in Spain (Esteruelas et al., 2015) due to their capacity to increase the Wine Haze Risk.

Another reason why among the winemakers is increasing the concern about how maturity affects parameters is the global warming. The climate change with the increasing of the average temperature, increment of CO_2 in the atmosphere and lower availability of chilling unit accumulation for the vines, is responsible for an anticipation of phenology and a decoupling among the accumulation of different compounds such as sugar and phenolic compounds (Jones et al. 2005). Obviously global warming implies even more other alteration of the grape berry composition. As shown by Buttrose et al. (1971) temperature influences many components of grape development, including for instance the breakdown of acids and berry colour development. In particular, temperatures above 30 °C for prolonged periods can induce heat stress, which leads to premature veraison, berry abscission, enzyme inactivation and reduced flavour development (Buttrose et al., 1971; Kliewer, 1977).

Furthermore, ripening grape berries are designed to minimise transpirational water loss (Radler, 1965; Possingham et al., 1967; Blanke et al., 1999; Rogiers et al., 2004) thus they are not able to protect themselves from overheating by evaporative cooling mechanism as it is for the leaves. Thus, while high temperatures tend to accelerate grape ripening, heat waves will quickly lead toward inhibition or even denaturation of berry proteins, and to symptoms of sunburn (Webb et al., 2008 a, b).

Protein haze in wine is currently avoided by removing the grape juice proteins before or after the alcoholic fermentation by bentonite addition. As known bentonite entails many drawbacks (Van Sluyter et al., 2015). Thus in this work it has been also evaluated the use heat treatment and heat treatment coupled with two different enzymes in order to eliminate or at least reduce the need of bentonite in winemaking process. In particular, this study demonstrated that there is the possibility to use proteases as an efficient treatment to control the WHR of Sauvignon Blanc wines. Nevertheless, the efficiency of the enzyme was minimal in Sauvignon Blanc because the heat treatment was already able to lead toward the production of wines completely stable from the colloidal point of view and furthermore, when protease has been applied without heating it has led to a wine richer in protein and even more susceptible to protein haze risk. Thus, further investigations are already planned for the coming vintages, to see if it is possible to confirm these first conclusions with wines/grape juices from different varieties, at different levels of maturity and produced in different areas (Alsace, val de la Loire).

Moreover, it would be interesting to evaluate the effect of such treatments on wine sensory qualities point of view, especially in order to estimate the impact of the heat treatments. However, thanks to the improved understanding of the mechanisms of haze formation, in Spain new alternative methods of protein stabilization are being investigated by the wine industry among one of the most promising solutions seems to be properly the use of enzymes. Other proteases are also currently being investigated that are active at winemaking temperatures and are specific against grape haze-forming proteins. For example, aspergillo-pepsin were

used in a previous study in Australia dedicated to grape juice and wine proteins from Chardonnay Blanc and Sauvignon Blanc wines with positive results (Marangon et al., 2012). Further studies surely will allow the development of better predictive tools for haze potential prevention and more targeted techniques possibly by utilizing those proteases that are active at winemaking temperature, will benefit wines, winemakers and customers worldwide (Steven et al., 2015).

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